

## Exposure of Tyrosine Residues in Protein. Reaction of Cyanuric Fluoride with Ribonuclease, $\alpha$ -Lactalbumin, and $\beta$ -Lactoglobulin\*

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**ABSTRACT:** The state of tyrosine residues (accessible or buried) in ribonuclease,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin has been examined with cyanuric fluoride. Ribonuclease was found to contain three reactive and three unreactive residues, the reactive ones being of different degrees of reactivity.  $\alpha$ -Lactalbumin contains four re-

active residues and one unreactive residue. The reactive residues are of two types, three being of the same degree of reactivity, the fourth being less reactive.  $\beta$ -Lactoglobulin contains three reactive residues and one unreactive residue. The reactive ones can be subdivided into two residues of greater and one of lower reactivity.

**A**nomalous tyrosine ionization behavior in proteins was first observed in ovalbumin by Crammer and Neuberger (1943), who concluded that tyrosine residues in ovalbumin are not free to ionize in the native state due to restrictions imposed upon the protein configuration by its tertiary structure. Since then it has become generally accepted to regard the idiosyncrasies of tyrosine behavior in proteins as reflections of the secondary and tertiary structures of the proteins (Beaven, 1961; Wetlaufer, 1962). The forces which prevent ionization of tyrosine residues in native proteins are believed to be either hydrogen bonds to specific acceptor groups, as first suggested by Crammer and Neuberger (1943), or hydrophobic forces which cause uncharged tyrosine side

chains to be buried in the protein interior, as proposed by many authors (Yanari and Bovey, 1960; Williams and Foster, 1959; Tanford, 1962), or a combination of the two effects (Edsall, 1963).

It is customary to divide the tyrosine residues of proteins into two broad classes, normal and buried. Normal tyrosine residues are very close to simple tyrosine peptides in their ionization behavior; they are assumed to be completely exposed to solvent molecules. Buried tyrosine residues do not resemble simple tyrosine peptides in their ionization behavior and are assumed to be prevented from intimate contact with solvent molecules (Beaven, 1961; Wetlaufer, 1962; Edsall, 1963).

In reality, a rigorous classification into completely exposed and completely buried residues is a great oversimplification, because frequently one is dealing with residues of an intermediate class, neither fully exposed nor fully buried. This fact has been stressed repeatedly by Laskowski (1966) and elegantly demonstrated for the case of tryptophan exposure in  $\alpha$ -chymotrypsinogen and lysozyme (Williams and Laskowski, 1965; Williams *et al.*, 1965).

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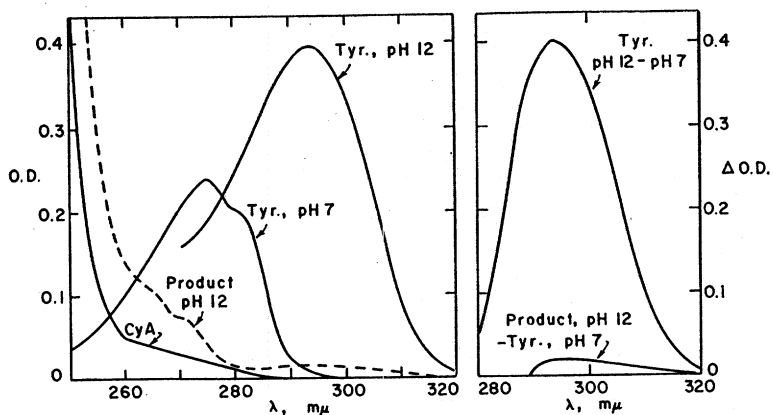


FIGURE 1: Direct and difference ultraviolet spectra of tyrosine at pH 7 and 12, cyanuric acid, and the reaction product between tyrosine and cyanuric fluoride. Tyrosine concentration,  $1.8 \times 10^{-4}$  M, CyF concentration, 0.232 M.

The investigation of tyrosine residues in proteins has been widely used as a probe of secondary and tertiary structures; the principal approaches include spectrophotometric and electrometric titration, solvent perturbation spectroscopy, and chemical modifications. Recently, *N*-acetylimidazole has been reported as a suitable reagent for the determination of exposed tyrosine residues (Riordan *et al.*, 1965).

When the results of titration, solvent perturbation, and reaction with *N*-acetylimidazole are compared, as far as this is possible, it is found that the first is probably the least selective. *N*-Acetylimidazole, as a diagnostic reagent for normal tyrosine residues, shows close agreement with the titration data. There are, however, apparent discrepancies which might indicate that this reagent is more sensitive than hydrogen ions (Riordan *et al.*, 1965; Perlmann, 1966).

In 1963, Kurihara *et al.* introduced cyanuric fluoride (CyF)<sup>1</sup> as a new tyrosine-modifying reagent. They observed that the ultraviolet absorption of tyrosine was greatly reduced and the position of the 278-mμ absorption band was shifted to shorter wavelength upon treatment with cyanuric fluoride. Other amino acids were found to be either not affected or affected only in the region below 290 mμ. CyF may be considered as a very reactive acid halide; in complete analogy to the acetylation of phenols with acid halides, cyanurylation results in the corresponding oxygen derivative of tyrosine, as has been shown in studies using tyrosine and its derivatives.<sup>2</sup> Since the reaction product of tyrosine with CyF, as well as cyanuric acid, which is the hydrolysis product of cyanuric fluoride, do not absorb in the ultraviolet region above 290 mμ to any significant extent (about 3%) as shown on Figure 1, a procedure for the application of this reagent to proteins was devised and the states of the tyrosine residues in insulin (Kurihara *et al.*, 1963; Aoyama *et al.*, 1965), lysozyme (Kurihara *et al.*, 1963), and  $\alpha$ -chymotrypsin (Hachimori *et al.*, 1965) were

studied. The results of these studies are shown in Table I and compared with those of the reaction with *N*-acetylimidazole and solvent perturbation. These data show that for  $\alpha$ -chymotrypsin and lysozyme the results obtained with CyF are in complete agreement with those of titration, reaction with *N*-acetylimidazole, and solvent perturbation. Insulin, however, presents a different and very interesting picture. Here, CyF distinguishes between three types of tyrosine residues: two residues which react at pH 9, one residue which becomes reactive at pH 12, and one residue which becomes reactive only after exposure for 3 hr to pH 12.5.<sup>3</sup>

The results of Table I suggest that CyF must be quite sensitive to the variation in the environment of tyrosine residues and that it is capable of detecting a much more subtle gradation in the reactivity of tyrosine residues than is discernible with *N*-acetylimidazole. This in turn is more discriminating than titration techniques (Riordan *et al.*, 1965; Perlmann, 1966). The states of the tyrosine residues in ribonuclease,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin have been examined with cyanuric fluoride as probing agent, and the results are reported in this paper.

#### Experimental Procedure

**Materials.** Cyanuric fluoride, purchased from Hynes Chemical Research Corp.,<sup>4</sup> was distilled before use. Dioxane, best grade from Fisher Scientific Co., was distilled twice from potassium hydroxide pellets and stored in the frozen state. Ribonuclease was five-times-crystalline bovine pancreatic ribonuclease from Mann Research Laboratories, Inc.  $\alpha$ -Lactalbumin was the gift of Dr. W. G. Gordon and Dr. M. J. Kronman.  $\beta$ -Lacto-

<sup>3</sup> In the present study it was not found possible to react this last residue. This might be due to the fact that the reaction was carried out at 3° rather than at 25° as was probably the case in the report of Kurihara *et al.* (1963).

<sup>4</sup> It is not implied that the U. S. Department of Agriculture recommends the above company or its products to the exclusion of others in the same business.

TABLE 1: States of Tyrosine Residues in  $\alpha$ -Chymotrypsin, Lysozyme, and Insulin.

Protein	Titration No. of Normal Groups	N-Ac-Im Reactive Groups	Solvent Perturbation Technique	CyF Reactive Groups	Ref
$\alpha$ -Chymotrypsin	1 + 1	2	—	2	<i>a-c</i>
Lysozyme	2	2	2 exposed	2	<i>a,d-f</i>
Zn-insulin	4 3	4	2 exposed	2 + 1 + 1	<i>a,b,g,f</i> <i>h</i>

<sup>a</sup> Tanford (1962). <sup>b</sup> Riordan *et al.* (1965). <sup>c</sup> Hachimori *et al.* (1965). <sup>d</sup> M. J. Kronman, private communication. <sup>e</sup> Williams *et al.* (1965); Williams and Laskowski (1965). <sup>f</sup> Kurihara *et al.* (1963). <sup>g</sup> Weil *et al.* (1965); in Zn-free insulin three groups are found to be exposed. <sup>h</sup> Inada (1961).

globulin was six-times-crystallized  $\beta$ -lactoglobulin A prepared from the milk of individual cows and the gift of Dr. R. Townend.

**Methods.** Spectroscopic measurements were made at room temperature on a Cary Model 14 recording spectrophotometer. The pH measurements were made at room temperature with a Beckman Zeromatic Model II pH meter. Protein concentrations were determined in 0.1 M phosphate buffer (pH 7.0) containing 10% dioxane from the extinction coefficients at 278 m $\mu$  for ribonuclease, using  $\epsilon$  9700 (Sage and Singer, 1962); at 280 m $\mu$  for  $\alpha$ -lactalbumin with an absorptivity of 20.1 dl/cm g (Kronman and Andreotti, 1964) and at 278 m $\mu$  for  $\beta$ -lactoglobulin using an absorptivity of 9.6 dl/cm g (Townend *et al.*, 1960a).

Spectrophotometric titrations were carried out in 1 M KHCO<sub>3</sub> buffers containing 10% dioxane by the difference spectral technique (Wetlaufer, 1962). The ultraviolet spectra were recorded against protein solutions dissolved in a 0.1 M phosphate buffer (pH 7.0), immediately after mixing and at 10-min periods thereafter. The optical density after 60 min was taken as the final value, since there was no further increase with time. Usually the final value of the optical density was attained after 45 min. The maxima of the difference spectra were found to be at 295.0 m $\mu$  for ribonuclease, 299.0 m $\mu$  for  $\alpha$ -lactalbumin, and 297.5 m $\mu$  for  $\beta$ -lactoglobulin. The number of ionized tyrosine residues in ribonuclease was calculated using an extinction coefficient of 2630 for the phenoxide ion (Tanford *et al.*, 1955); for  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, the corresponding extinction coefficient was taken as 2300 (Crammer and Neuberger, 1943; Sage and Singer, 1962).

**Reaction with Cyanuric Fluoride.** The basic procedure for the cyanurylation of proteins may be summarized as follows. A protein solution is treated with an excess of CyF at the pH of interest. The reaction mixture is then allowed to stand at a given temperature for an arbitrary length of time, sufficiently long to assure that all of the unreacted CyF has become hydrolyzed to the level of cyanuric acid. This time is not related to the time necessary for the completion of the reaction between CyF and

the reactive ("available") tyrosine residues. This reaction is very rapid; it must take place before the occurrence of any considerable hydrolysis of CyF. Since the latter process occurs quite rapidly (CyF is hygroscopic), the reaction of CyF with the tyrosine residues must be terminated immediately after mixing of the two solutions. The complete hydrolysis of the last traces of CyF to cyanuric acid is essential if spectral interference in the region of 290–300 m $\mu$  is to be precluded.

Upon completion of the above-described steps, the reaction mixture is adjusted to pH 13 and the ultraviolet spectrum is recorded between 290 and 340 m $\mu$  again after standing at room temperature for a given period. An aliquot of the reaction mixture is simultaneously adjusted to pH 7 and used as a reference. If it is found that treatment with CyF does not change the ultraviolet absorption of the protein at pH 7 between 290 and 340 m $\mu$ , the pH 7 CyF-treated reference solution may be replaced by a pH 7 solution of unreacted protein at an identical concentration.

The time selected for recording the ultraviolet spectra is dictated by the results of an independent spectrophotometric titration of the untreated protein. It is the time necessary for the complete ionization of all buried tyrosine residues, *i.e.*, the time necessary for a sufficient unfolding of the protein that would permit these groups to come in contact with the medium and become ionized. In a CyF-treated protein only those tyrosine residues which had not reacted with CyF under given conditions of pH and temperature will ionize. Thus, the number of tyrosine residues which did react with CyF under a given set of conditions is given by the difference between the numbers of tyrosine residues which can be caused to ionize after standing at pH 13 in the untreated and CyF-treated proteins.

The detailed experimental procedure used in the studies on ribonuclease,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin follows. Protein solution (1 ml) in 0.1 M phosphate buffer (pH 7.0) was mixed with 6 ml of 1 M KHCO<sub>3</sub> buffer of a given pH. To this 1 ml of CyF of a given concentration (made up by dissolving freshly distilled CyF in dry dioxane) was added and the reaction

flask was shaken well. After standing for 1.5 hr at 25° (or 3°), the pH of the reaction mixture was adjusted to pH 13 by adding 10 M KOH and made up to 10 ml with 1 M KHCO<sub>3</sub> buffer (pH 13). The ultraviolet spectrum was recorded *vs.* a standard of the same concentration in 0.1 M phosphate buffer (pH 7.0) containing 10% dioxane. Ribonuclease was used at a concentration of 6 mg/ml, while  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin were at 10 mg/ml.

In the case of ribonuclease and  $\beta$ -lactoglobulin, there was no difference in ultraviolet absorption in the 290–340-m $\mu$  region between untreated protein dissolved in a phosphate buffer (pH 7) containing 10% dioxane and CyF-treated protein adjusted to pH 7. In the case of  $\alpha$ -lactalbumin it was found that protein solutions in a phosphate buffer (pH 7) did not have the same absorption at 299 m $\mu$  as CyF-treated samples that had been adjusted to pH 7. Furthermore, the ultraviolet spectra of all CyF-treated samples showed a time-dependent drifting of the optical density. As a result, the ultraviolet spectra were recorded at 0, 15, 30, 45, and 60 min after final adjustment of pH and time plots of optical density *vs.* time were obtained for each combination of CyF concentration and pH used. Similar time plots of CyF-treated samples adjusted to pH 7 were used as correction factors for those experimental results which were based on difference spectra *vs.* untreated standards in phosphate buffer (pH 7). Furthermore, since aggregation can be expected to be a very serious problem in the case of  $\alpha$ -lactalbumin, a separate set of spectra for a variety of pH values and CyF concentrations was recorded *vs.* a pH 13 standard at 15–20 min after final adjustment of pH.

The number of moles of tyrosine residues which had reacted under any given conditions of pH, CyF concentration, and temperature was calculated as in the case of a spectrophotometric titration; the optical density at the wavelength of maximal absorption (295.0, 299.0, and 297.5 m $\mu$ ) was divided by the product of the molar concentration of protein with the molar extinction coefficient of phenoxide ion in the given protein. When the spectrum is measured against a pH 7 standard, this gives the number of moles of unreacted tyrosine residues. For the experiments recorded *vs.* a pH 13 standard, this calculation results in the number of reacted tyrosine residues, since what is recorded in this case is total tyrosine (pH 13 standard) less the tyrosine residues which had not reacted with CyF (reaction product adjusted to pH 13).

In the case of  $\alpha$ -lactalbumin, since the optical density was time dependent, three values of the optical density were taken instead of one; these were at 0, 15, and 60 min. Since, however, the ionization of  $\alpha$ -lactalbumin at pH 13 is complete only after 60 min, the 0- and 15-min data had to be corrected to the 60-min ionization values. This was done by using a standard ionization curve from which the degree of ionization at any given time could be estimated. This type of correction was also used for data obtained in runs *vs.* the pH 13 standard since these corresponded to an ionization time of 15–20 min.

Complete agreement was found between the number

of reacted tyrosine residues calculated from difference spectra *vs.* pH 7 phosphate standards and those based on difference spectra *vs.* pH 13 KHCO<sub>3</sub> standards. Moreover, in the case of  $\alpha$ -lactalbumin, good agreement was obtained between the 0-, 15-, and 60-min values calculated on the basis of difference spectra *vs.* pH 7 standards.

*Method of Data Analysis.* The tyrosine residues in a protein were classified on the basis of changes in their reactivity with CyF, caused by changes in pH, temperature, or both. This was done as follows. In preliminary experiments, the number of reactive tyrosine residues in a protein was determined at 25 and 3° as a function of pH. The protein and CyF concentrations were kept constant. This results in a pH profile for a given protein. On the basis of this profile, pH values close to whole numbers of reacted tyrosine were selected and concentration curves (experiments with increasing concentrations of CyF) were obtained at these pH values. Furthermore, at these pH values there was no time-dependent tyrosine ionization, either in the native or partly denatured states ( $\alpha$ -lactalbumin, pH 10.0;  $\beta$ -lactoglobulin, pH 10.8). According to the criteria established by Kurihara *et al.* (1963), a plateau in a concentration curve indicates that a limit has been reached with respect to tyrosine reactivity. On the other hand, the inability to reach such a plateau, which is usually accompanied by a wide scattering of results, poor reproducibility, and a noticeable increase in reactivity with an increase in concentration, indicates that the chosen conditions are not suitable as less exposed groups become partly modified at higher CyF concentrations. Near a plateau, concentration curves assume an asymptotic character. If the pH profiles for 25 and 3° are not the same, concentration curves must be run at both temperatures. The data for pH profiles should be plotted also as the relation between reactive tyrosines and ionized tyrosines to ascertain that reactivity is not simply a direct function of ionization. Each plateau is taken to mean a discrete state in tyrosine residue reactivity and the number of such plateaus gives the number of different types of tyrosine residues in any given protein (Kurihara *et al.*, 1963).

## Results

*Ribonuclease.* Ribonuclease contains six tyrosine residues, three of which are abnormal. At room temperature, exposure to pH values above 11.5 results in denaturation with the concomitant release to ionization of the previously buried tyrosines. The titration curve of ribonuclease in 1 M KHCO<sub>3</sub> buffer containing 10% dioxane showed no significant differences from that reported by Tanford *et al.* (1955) and Tanford and Hauenstein (1956).

Preliminary experiments with 0.232 M cyanuric fluoride, carried out at eight pH values between 9.5 and 12.2 at temperatures of 3 and 25°, have shown that, at 25°, the number of reactive tyrosine residues is 1.8 at pH 9.5, reaches 2.8 at pH 11.7, and does not exceed 3 up to pH 12.0; at 3° the respective values are 1.0, 1.7, and 2.4 groups. Thus, there is a constant difference of about one

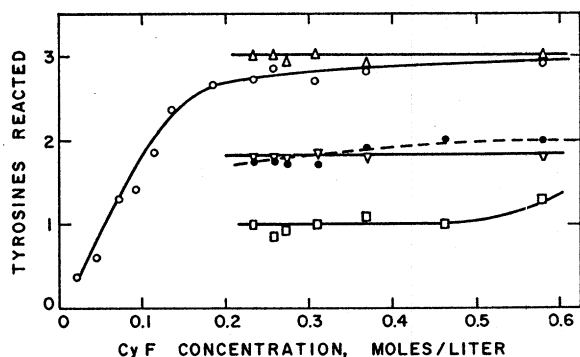


FIGURE 2: Dependence of ribonuclease tyrosine reactivity on CyF concentration. ( $\square$ ) 3°, pH 9.5. ( $\nabla$ ) 3°, pH 10.9. ( $\bullet$ ) 25°, pH 9.5. ( $\circ$ ) 25°, pH 10.9. ( $\Delta$ ) 25°, pH 11.2.

group (0.8 at pH 9.5 and 1.1 at pH 11.2) between the reactivity of tyrosine residues at the two temperatures in the pH range below denaturation.

Concentration curves, obtained at pH values close to whole numbers in tyrosine reactivity, are shown in Figure 2. At 3° and pH 9.5 a plateau is obtained at the level of one tyrosine. There is, however, a definite increase in tyrosine reactivity at the highest CyF concentration. At pH 10.9 a second plateau is observed at 1.8 tyrosine residues. At 25° the experiments were carried out at pH 9.5, 10.9, and 11.2. At pH 9.5 the number of reactive tyrosine residues is striving toward two and a plateau is reached at the highest concentrations. A second plateau of three groups is obtained at pH 11.2. The data at pH 10.9 are typical for a case where a plateau is being approached. The number of reactive tyrosine residues slowly increases with an increase in CyF concentration, approaching asymptotically the next plateau, and the points are more scattered.

Above the denaturation point, reaction of ribonuclease with CyF at pH values 11.7, 12.2, and 12.5 at 25° resulted in a plateau of three reactive tyrosine residues in all cases. Furthermore, experiments in which ribonuclease had been denatured by keeping it at pH 13 for either 1 or 3 hr at 25° and then treated with CyF at five different pH values between 10.0 and 12.4 always resulted in the reaction of exactly three tyrosines.

**$\alpha$ -Lactalbumin.**  $\alpha$ -Lactalbumin has five tyrosine and five tryptophan residues for a molecular weight of 15,500. The titration of  $\alpha$ -lactalbumin in 1 M  $\text{KHCO}_3$  buffer containing 10% dioxane indicates that four tyrosine residues ionize instantaneously below pH 11 with an apparent  $pK$  of 10.4. Above this pH the ionization becomes time dependent. At pH 13, ionization of the fifth group is completed within 1 hr. An average value of 4.84 tyrosine residues was obtained using  $\epsilon$  2300.

In preliminary experiments,  $\alpha$ -lactalbumin was treated with CyF at pH values between 9.0 and 12.0 at 3 and 25°. The number of reacted tyrosines under these conditions starts at 2.5 and gradually increases by one and one-half groups up to pH 10.0, where the maximum of

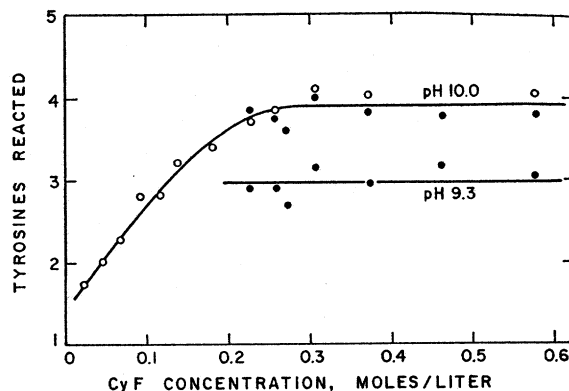


FIGURE 3: Dependence of  $\alpha$ -lactalbumin tyrosine reactivity on CyF concentration. ( $\circ$ ) pH 7 standard. ( $\bullet$ ) pH 13 standard.

four reactive groups is reached. The remaining fifth group cannot be reacted even by increasing the pH to 12. There was no difference in reactivity whether the experiments were carried out at 3 or 25°.

Below pH 10.0 the number of groups reacted exceeds that ionized by three (pH 10.0: one group ionized, four reacted; pH 9.3: three groups reacted, 0.1 ionized). Since it is known (Kronman *et al.*, 1966) that conformational changes occur at close to pH 9.5, these two points can be taken as representative of  $\alpha$ -lactalbumin in the native and expanded states (Kronman *et al.*, 1966). On the basis of these observations, concentration curves were obtained at pH values 9.3 and 10.0 and the results are shown in Figure 3. Native  $\alpha$ -lactalbumin displays a plateau at three groups, while expansion of the molecule releases an additional tyrosine to reactivity, the plateau being obtained at four groups.

**$\beta$ -Lactoglobulin.**  $\beta$ -Lactoglobulin contains four tyrosine and two tryptophan residues per chain of 18,000 mol wt. Titration in 1 M  $\text{KHCO}_3$  buffer containing 10% dioxane indicates that three tyrosine residues titrate with a single apparent  $pK$  below pH 12, while the last group ionizes only slowly at pH 13 (Figure 4A), ionization being complete after 1 hr. These results agree quite well with those of Tanford and Swanson (1957) and Nozaki *et al.* (1959) below pH 12. No valid comparison can be made for the region above pH 12, since these authors did not examine the region close to pH 13. They did report, however, the presence of a second conformational change above pH 12 (the first one occurring at pH 9.7). Pantaloni (1965) has found one abnormal tyrosine residue on the basis of differential spectrophotometry between aqueous and alcoholic solutions.

Preliminary experiments on  $\beta$ -lactoglobulin were carried out at 3° at pH values between 9.0 and 12.6, and the results are shown in Figure 4B. The number of reactive groups was found to start at about 1.8, reach three at pH 10.8, and remain at three up to pH 12.6. Experiments carried out on denatured material (1-hr exposure to pH 13 at room temperature) under identical conditions gave the same results.

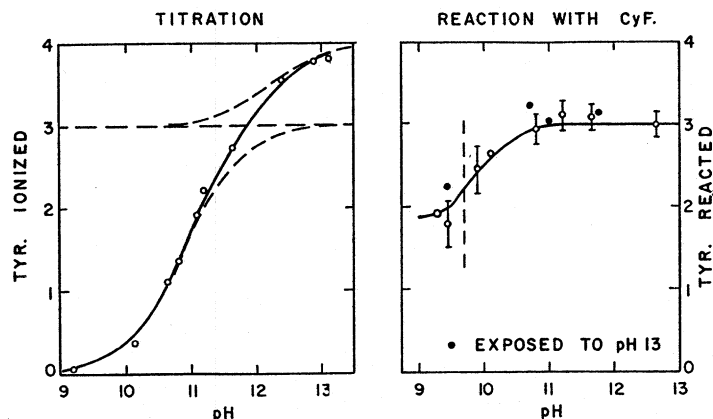


FIGURE 4:  $\beta$ -Lactoglobulin tyrosines. (A) (left) Titration in 1 M bicarbonate buffer. The dashed lines are calculated curves for the ionization of three groups with an apparent  $pK$  of 10.9 and one group with an apparent  $pK$  of 12.2. The electrostatic interaction parameter,  $w$  (Tanford, 1962), is zero in both cases. The solid line represents the sum of the two calculated curves. Circles are experimental points. (B) (right) Dependence of tyrosine reactivity on pH at 3°, 0.232 M CyF.

Since above pH 9.7  $\beta$ -lactoglobulin is known to undergo gradual denaturation, pH values 9.3 and 10.8 were selected to represent the native and partly denatured states. A concentration curve was run at 3° at pH 9.3. This gave erratic results indicating between 1.5 and 2 reactive tyrosine residues, the plot of the number of reactive tyrosine residues as a function of CyF concentration slowly approaching two groups. A plateau of two groups was obtained by carrying out the same experiments at 25° under rigorous temperature control (Figure 5). A plateau at three groups was obtained at pH 10.8 at both 3 and 25° (Figure 5), the tyrosine reactivity being identical at the two temperatures. The observation that CyF treatment of samples partly denatured by exposure to pH 13 results in the same number of reactive groups as in the native protein at identical pH values is consistent with the reported partial reversal of optical rotation when alkali-denatured  $\beta$ -lactoglobulin is slowly returned to the pH zone in which the protein is normally native (Herskovits *et al.*, 1964).

#### Discussion

The reaction of ribonuclease with cyanuric fluoride shows that no more than three tyrosine residues are available to this reagent either in the native or in the alkali-denatured state. It seems reasonable to equate these reactive residues with the three found to be normal by titration and to be reactive with *N*-acetylimidazole (Riordan *et al.*, 1965). The present study suggests, furthermore, that these normal residues are not identical, as is generally believed.

The gradation in the reactivity of the normal tyrosine residues can be brought out by the appropriate combination of pH and temperature of reaction. At 3° and pH 9.5 only one tyrosine residue is reactive. Raising the temperature to 25° or changing the pH to 10.9 results in the reaction of a second residue. The difference in reactivity

between these two groups must be quite small, however, as evidenced by the upturn in the concentration curve at the highest CyF concentration used at pH 9.5 and 3°. The third group becomes reactive when the pH is raised above 11, but only at room temperature. The effect of temperature is very striking, since a change of the temperature from 3 to 25° at any pH results in an increase in reactivity by one tyrosine residue. This cannot be attributed to the difference in ionization at the two temperatures, since it has been shown by Tanford *et al.* (1955) that between pH 9.5 and 11.5 the ionization increases by only 0.2–0.3 group when the temperature is raised from 6 to 25°. Thus, on the basis of their reactivity with CyF, the normal tyrosine residues of ribonuclease appear to be of three distinct types. Two are rather close in their reactivity, while the third one is considerably less reactive. Although a voluminous literature exists on the differences between the abnormal tyrosine residues of ribonuclease (Scheraga and Rupley, 1962),

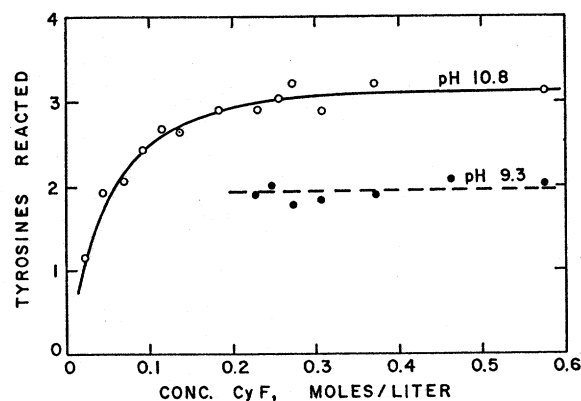


FIGURE 5: Dependence of  $\beta$ -lactoglobulin tyrosine reactivity on CyF concentration at 25°.

there are hardly any reports of similar nature concerning the normal residues. Solvent perturbation studies show that only 40% of the tyrosine residues of ribonuclease are exposed and accessible to perturbant to varying degrees<sup>5</sup> (Herskovits and Laskowski, 1960). This implies that one of the normal tyrosine residues is not fully exposed to solvent, a situation similar to what has been found with bovine serum albumin (Herskovits and Laskowski, 1962). Such a residue should be sensitive to the size of the probing reagent. Denaturation with ethylene glycol brings the number of exposed groups exactly to 50% (Herskovits, 1965). Furthermore, enzymatic work on ribonuclease suggests that the normal tyrosine residues are neither completely free nor all identical. Thus, native ribonuclease cannot be oxidized with polyphenol oxidase (Lissitzky *et al.*, 1960), while only one tyrosine residue is oxidized by tyrosinase (Yasunobu and Dandliker, 1957).

Recently, Friedman *et al.* (1966) have reported that iodination of ribonuclease at pH 6.7 and 4° with 2, 4, and 6 moles, respectively, of iodine/mole of ribonuclease gave di-, tetra-, and hexaiodinated derivatives as major components. The hexa- and tetraiodinated derivatives contained three iodinated tyrosines (residues 73, 76, and 115), while the diiodinated derivative contained only two (residues 73 and 76). These data indicate quite clearly that the three normal residues cannot be identical in their environment and, thus, exposure to solvent.

The lack of reactivity of the three abnormal tyrosine residues after exposure of the protein to pH 13 is not surprising. Of the six tyrosines in ribonuclease, two are located next to cysteines in the primary structure of the protein, while a third one is one residue removed from an SS bridge (Smyth *et al.*, 1963). Such a situation may contribute to steric hindrance in a reaction involving a bulky reagent. This effect would be of the same nature as that observed by Williams *et al.* (1965) in the solvent perturbation spectroscopy of ribonuclease, as well as other proteins, even in the presence of 8 M urea; these authors have concluded that disulfide bonds impede accessibility of reagents to adjacent residues. Further, solvent perturbation studies have shown (Herskovits, 1965) that, even after denaturation in 8 M urea or in 2-chloroethanol, only four tyrosines are available to contact with the perturbant, dimethyl sulfoxide, while denaturation with ethylene glycol does not release any of the three abnormal residues to contact with solvent. Finally, titration experiments on ribonuclease, denatured in 7 M guanidine hydrochloride, indicate that the six tyrosines still titrate in two groups of three residues each, with slightly different pK values (Nozaki and Tanford, 1967). It would appear only reasonable that groups which display different degrees of accessibility to hydrogen ions and which are inaccessible to contact with a solvent component will not be capable of forming covalent bonds with a bulky reagent, such as cyanuric fluoride. One should point out that exposure to pH 13 results in a lower degree of unfolding of ribonuclease than is ob-

tained by treatment with 8 M urea or 7 M guanidine: at 6°, the three abnormal tyrosines are only partially titrated, even at pH 14 (Tanford *et al.*, 1955).

$\alpha$ -Lactalbumin is shown by the CyF reaction to have four reactive residues and one unreactive residue. Considering that the titration curve indicates the presence of one abnormal tyrosine residue, it is assumed that the CyF unreactive residue is the abnormal one. The CyF reactive groups are of two types: three are accessible in the native state, the fourth becoming accessible only after a conformational change. The difference in reactivity of the normal tyrosine residues receives some support from enzymatic studies. It has been reported that polyphenol oxidase attacks only three tyrosine residues (Lissitzky *et al.*, 1960) while tyrosinase oxidizes all five (Yasunobu and Dandliker, 1957). On the other hand, reaction with *N*-acetylimidazole shows four normal groups,<sup>6</sup> in complete agreement with the titration data. Therefore, the classification of the normal tyrosine residues in  $\alpha$ -lactalbumin into two types, as revealed by CyF, is quite consistent with other known facts. It is fair to assume that the three more reactive residues are the three polyphenol oxidase sensitive ones, while the fourth, somewhat less reactive residue, is among the four which are accessible to *N*-acetylimidazole.

It is interesting to note that in the case of  $\alpha$ -lactalbumin, just as in the case of ribonuclease, tyrosinase appears to be the more active enzyme, suggesting that it is less sensitive to the environment of the tyrosine residues than polyphenol oxidase. Furthermore, it seems significant that in  $\alpha$ -lactalbumin the reactivity of the groups with CyF does not differ whether the temperature is 3 or 25°, a situation different from that found with ribonuclease. This would suggest that in  $\alpha$ -lactalbumin a change in temperature does not induce any significant changes in the protein conformation neighboring to the tyrosine residues, while in ribonuclease the known change in conformation with change in temperature (French and Hammes, 1965) must loosen the structure of the molecule close to the normal tyrosines.

Of the four tyrosine residues in  $\beta$ -lactoglobulin, only three are reactive with CyF. Since titration indicates the presence of one abnormal tyrosine residue, this one is assigned as the one unreactive with CyF. The three normal residues are of two types. Two residues react with CyF below the onset of irreversible denaturation (pH 9.7), while the last one becomes reactive only when irreversible processes had set in. Below the point of denaturation, the reactivity is affected by a change in temperature from 3 to 25°. It seems of interest that Tanford and Taggart (1961) have described a conformational transition with a positive enthalpy in the pH range just below the onset of denaturation.

The reactivity of the third group above pH 10.5 can be related either to the partial irreversible unfolding of this molecule (Timasheff *et al.*, 1966) or to the dissociation of the  $\beta$ -lactoglobulin dimer into two single chains (Townend *et al.*, 1960b) at alkaline pH values (Georges

TABLE II: States of Tyrosine Residues in Ribonuclease,  $\alpha$ -Lactalbumin, and  $\beta$ -Lactoglobulin.

Protein	No. of Rev Titrating Groups	No. of Irrev Groups	pK	N-Acetyl- imidazole Reactive	Solvent Perturbation Technique	Tyrosinase <sup>e</sup>	Poly- phenol Oxidase <sup>f</sup>	CyF Reactive
Ribonuclease	3	3	9.9 <sup>a</sup>	3 <sup>b</sup>	40% exposed <sup>d</sup>	1	0	1 + 1 + 1
$\alpha$ -Lactalbumin	4	1	10.4	4 <sup>c</sup>	—	5	3	3 + 1
$\beta$ -Lactoglobulin	3	1	10.9 <sup>a</sup>	4	—	0	—	2 + 1

<sup>a</sup> Tanford (1962). <sup>b</sup> Riordan *et al.* (1965). <sup>c</sup> M. J. Kronman, private communication. <sup>d</sup> Herskovits and Laskowski (1960); see also footnote 5. <sup>e</sup> Yasunobu and Dandliker (1957). <sup>f</sup> Lissitzky *et al.* (1960). <sup>g</sup> Midpoint of three group ionization.

and Guinand, 1960), or a combination of both. Thus, the third normal residue could be located within the area of contact between the two subunits in the native protein and released to contact with solvent only upon molecular dissociation; in this case, burial would be equated to molecular aggregation and be independent of secondary and tertiary structure. The studies of the alkaline denaturation of this protein by Pantaloni (1965) would suggest, however, that this group is actually present within a crevice which must be enlarged before admitting CyF. This position is further supported by titration experiments<sup>7</sup> which indicate that the availability of the third group to hydrogen ions is little affected by changes in ionic strength from 0.05 to 2.0; it can be expected that the higher ionic strength would interfere strongly with the dissociation, which is driven by non-specific electrostatic repulsion (Townend *et al.*, 1960b; Timasheff, 1964).

It should be mentioned that all four tyrosine residues of  $\beta$ -lactoglobulin react with *N*-acetylimidazole.<sup>8</sup> This result, which is seemingly inconsistent with titration data, can serve as an example (see also Riordan *et al.*, 1965) of the complexity of studies aimed at the elucidation of the environment of individual tyrosine residues in a given protein.

The fact that even exposure to pH 13 with subsequent lowering of pH does not render reactive the last tyrosine residue can be related to the circular dichroism observation (Timasheff *et al.*, 1967) that increase in pH only gradually alters the conformation of  $\beta$ -lactoglobulin, with structured areas remaining evident even at quite high pH values. Furthermore, a decrease in pH is accompanied by a considerable reversal of the optical rotatory properties (Herskovits *et al.*, 1964), indicating the re-forming of some structure.

The general lack of reactivity with CyF of some tyrosine residues after exposure to high pH can serve as a further indication of the preservation of some structure

even after such drastic treatment. The cases of ribonuclease and  $\beta$ -lactoglobulin have been discussed already.  $\alpha$ -Lactalbumin is also known to undergo only a gradual conformational change as the pH is raised to high values (Kronman *et al.*, 1966). The difficulty with which tyrosine groups become exposed by an increase in pH is a rather widespread phenomenon; suffice it to cite as an example the situation which exists in the titration of human carbonic anhydrase (Riddiford *et al.*, 1965). As another example, one can cite the reaction of stem bromelain with CyF (Tachibana and Murachi, 1966) which gives seven to eight groups reactive below pH 10.2, 13–14 groups reactive at pH 12.1, with five to six groups being inaccessible; the titration of this protein indicates nine normal tyrosine residues, seven ionizing with a pK of 11.9, one with a pK of >12, and one residue still not ionized at pH 13.

In Table II the results of these studies are summarized and compared with information available from the literature (see also Table I). It is evident that CyF is a highly sensitive tyrosine residue modifying reagent. In the cases of proteins for which there are comparable data, CyF appears to be somewhat more discriminating than *N*-acetylimidazole, being able to reveal subtle gradations of reactivity among normal tyrosine residues. This greater selectivity of CyF is probably related to its greater spatial requirements. Thus, if a tyrosine residue is located in a small crevice and is accessible to water molecules and hydrogen ions, it may also be accessible, although to different extents, to modifying reagents, depending on their size and the steric requirements of their transition states. Conformational changes, which result in the loosening of the protein structure around the residues in question, may broaden the molecular crevices to the point that they can accommodate bulkier substituents. In this respect, the sensitivity of cyanuric fluoride to the environment of tyrosine residues in proteins may be compared to that of solvent perturbation spectroscopy, using perturbants of various sizes (Laskowski, 1966). A particularly fruitful approach seems to be one in which the environment of tyrosine residues in proteins is probed by titration and reactivity with very re-

<sup>7</sup> J. J. Basch and S. N. Timasheff, private communications.

<sup>8</sup> M. J. Gorbunoff, unpublished results.



active (low selectivity) reagents, followed by reactivity with CyF under various conditions at which the residues in question become progressively more available as the secondary structure of the molecule is gradually loosened.

Finally, a reminder seems desirable that the cyanuric fluoride reaction is carried out in the presence of 10% dioxane. Thus, conclusions on the availability of tyrosine residues in a native protein involve the assumption that no conformational changes are induced by 10% dioxane. This is known to be true for ribonuclease (Weber and Tanford, 1959; Bigelow and Krenitsky, 1961) and  $\beta$ -lactoglobulin (Tanford *et al.*, 1960).

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#### Added in Proof

Since the submission of this paper, Robbins *et al.* (1967) have reported the titration curve of  $\alpha$ -lactalbumin, with the conclusion that all five tyrosine groups ionize normally in 0.15 M KCl.

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